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The Application of Product Inhibition Studies to One Substrate–One Product Enzymic Reactions

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A graphical procedure is presented for distinguishing between possible mechanisms of one substrate–one product enzymic reactions.

The conventional method for studying steady-state kinetics of one substrate–one product reactions is to measure the initial steady-state velocity in a reaction mixture where no product is present at the beginning of the reaction. If a plot of the reciprocal of this velocity against the reciprocal of the initial substrate concentration is linear, it is usually assumed that the mechanism of Peller & Alberty (1959) applies (for notation see below):

$$E+S \rightleftharpoons X_1 \rightleftharpoons X_2 \rightleftharpoons \ldots \rightleftharpoons X_i \rightleftharpoons \ldots \rightleftharpoons X_n \rightleftharpoons E+P \qquad \text{(Mechanism I)}$$

However, such a plot is also consistent with Mechanism II:

$$E+S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} X \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} F+P$$

$$F \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E \qquad \text{(Mechanism II)}$$

Further experiments to distinguish between these two mechanisms are rarely performed.

One of the most useful methods for distinguishing between possible mechanisms of enzyme action has been the application of product inhibition studies (see review by Cleland, 1970). However, the usual methods of product inhibition studies cannot be applied to one substrate–one product reactions, as is shown below.

Notation

The notation used is as follows:

E: a particular conformational form of an enzyme.

F: a conformational form of the enzyme distinct from E.

$[E_0]$: total initial concentration of enzyme; this includes all free species of the enzyme, but not complexes with substrate or product.

X: enzyme intermediate; when several intermediates are involved in a mechanism, these are denoted by X_1, X_2, X_3, ...

P and [P]: product of a reaction and its steady-state concentration.

$[P_0]$: initial product concentration.

S and [S]: substrate of a reaction and its steady-state concentration.

$[S_0]$: initial substrate concentration.

v: steady-state velocity.

v_0: initial steady-state velocity when both substrate and product are present in the reaction mixture at the start of a reaction; I treat v_0 as positive when the reaction goes in the forward (S → P) direction and as negative when it goes in the reverse (P → S) direction.

$(v_0)_{[P]=0}$: initial steady-state velocity when product is absent from the reaction mixture at the start of a reaction.

$(v_0)_{[S]=0}$: initial steady-state velocity when substrate is absent from the reaction mixture at the start of a reaction.

$\Delta v_0 = (v_0)_{[P]=0} - v_0$.

k: velocity constant; when several velocity constants are involved in a mechanism, these are denoted by k_{+1}, k_{-1}, k_{+2}, k_{-2}, k_{+3}, k_{-3} ...

Further symbols are defined as they appear in the text.

Product Inhibition Studies

In product inhibition studies the initial steady-state velocity is measured when one of the products, as well as the substrates, is present at the start of the reaction (Alberty, 1958). For such studies it is convenient to plot the reciprocal of the initial steady-state velocity against the reciprocal of the initial concentration of one of the substrates in mixtures where the initial concentrations of all other components are held fixed. Wong & Hanes (1962) have stated that such a plot is linear for mechanisms in which the substrate, the concentration of which is varied, combines

with only one enzyme species (free enzyme or enzyme intermediate).

When a reaction yields more than one product, all the negative terms in the steady-state rate equation contain a common factor, which is the mathematical product of the steady-state concentrations of all the products of the reaction (Wong & Hanes, 1962). Since, in product inhibition studies, only one product is present at the start of the reaction, all the negative terms in the steady-state rate equation disappear if we replace the steady-state concentrations of the substrates and products by their initial concentrations. Thus the analysis of such reactions involves initial steady-state equations that contain positive terms only.

This method cannot be used for reactions in which a single substrate is converted into a single product. If only one product is formed in a reaction, the negative terms in the steady-state rate equation do not disappear when the steady-state concentrations of the substrate and the product are replaced by their initial concentrations. When the initial concentration of product is held fixed, a plot of the reciprocal of the initial steady-state velocity against the reciprocal of the initial substrate concentration is non-linear, even for mechanisms in which the substrate combines with only one enzyme species.

Kinetic Analysis of One Substrate–One Product Reactions

The steady-state rate equation for Mechanism I can be written as:

$$v = \frac{\frac{V_s}{K_s}\cdot[S] - \frac{V_p}{K_p}\cdot[P]}{1 + \frac{[S]}{K_s} + \frac{[P]}{K_p}} \qquad (1)$$

for $n = 1, 2, 3, \ldots$ (Peller & Alberty, 1959). V_s and K_s are kinetic parameters obtained from experiments in which only substrate is present at the start of the reaction; V_s is the maximum value of $(v_0)_{[P]=0}$ that is approached as $[S_0] \rightarrow \infty$, and K_s is equal to the initial substrate concentration at which $(v_0)_{[P]=0} = V_s/2$. The kinetic parameters V_p and K_p are analogous to V_s and K_s, and are obtained from experiments in which only product is present at the start of the reaction. Expressions for these kinetic parameters in terms of the velocity constants of the mechanism are given by Peller & Alberty (1959).

Cleland (1963) and Taraszka & Alberty (1964) pointed out that the steady-state rate equation for Mechanism II has an additional term in [S][P] in the denominator, namely:

$$v = \frac{\frac{V_s}{K_s}\cdot[S] - \frac{V_p}{K_p}\cdot[P]}{1 + \frac{[S]}{K_s} + \frac{[P]}{K_p} + \frac{[S][P]}{K_{sp}}} \qquad (2)$$

where:

$$V_s = \frac{k_{+2}k_{+3}[E_0]}{k_{+2}+k_{+3}}$$

$$V_p = \frac{k_{-1}k_{-3}[E_0]}{k_{-1}+k_{-3}}$$

$$K_s = \frac{(k_{-1}+k_{+2})(k_{+3}+k_{-3})}{k_{+1}(k_{+2}+k_{+3})}$$

$$K_p = \frac{(k_{-1}+k_{+2})(k_{+3}+k_{-3})}{k_{-2}(k_{-1}+k_{-3})}$$

$$K_{sp} = \frac{(k_{-1}+k_{+2})(k_{+3}+k_{-3})}{k_{+1}k_{-2}}$$

Mechanisms I and II cannot be distinguished between by measuring $(v_0)_{[P]=0}$ or $(v_0)_{[S]=0}$ because:

(i) if the product is not present at the start of the reaction, eqns. (1) and (2) both simplify to:

$$(v_0)_{[P]=0} = \frac{V_s[S_0]}{[S_0]+K_s} \qquad (3)$$

(ii) If the substrate is not present at the start of the reaction, eqns. (1) and (2) both simplify to:

$$(v_0)_{[S]=0} = -\frac{V_p[P_0]}{[P_0]+K_p} \qquad (4)$$

(iii) the Haldane relationship (Haldane, 1930) for both mechanisms is:

$$K_{eq.} = \frac{V_s K_p}{V_p K_s} \qquad (5)$$

where $K_{eq.}$ is the equilibrium constant for the overall reaction $S \leftrightharpoons P$.

It is, however, possible to distinguish between Mechanisms I and II by measuring v_0 at saturating $[S_0]$, since the steady-state rate equation for Mechanism II contains, in the denominator, an [S][P] term that is absent from the equation for Mechanism I (Cennamo, 1969). From eqn. (1) it can be seen that:

$$\lim_{[S_0]\rightarrow\infty} v_0 = V_s \qquad (6)$$

whereas for eqn. (2):

$$\lim_{[S_0]\rightarrow\infty} v_0 = \frac{V_s}{1 + \frac{K_s}{K_{sp}}\cdot[P_0]} \qquad (7)$$

In theory, therefore, if a plot of $1/v_0$ (at saturating $[S_0]$) against $[P_0]$ is linear, then the results are consistent with Mechanism II; if v_0 (at saturating $[S_0]$) is independent of $[P_0]$, then the results are consistent with Mechanism I. In practice, however, it may be impossible to obtain measurements at sufficiently high values of $[S_0]$.

In the next section, I present a graphical procedure

for analysing experimental values for a one substrate–one product enzymic reaction when both substrate and product are present at the start of the reaction. With this technique it is possible to decide whether steady-state results for such a reaction are consistent with Mechanisms I or II. It also provides a simple method for estimating the parameter K_{sp} of eqn. (2).

Graphical Method for Distinguishing Mechanisms I and II

The method is based on eqns. (1) and (2). These may be converted into a more convenient form by introducing the term Δv_0, which is the difference between $(v_0)_{[P]=0}$ and v_0. For eqn. (1):

$$\Delta v_0 = \frac{K_s\{(V_s + V_p)[S_0] + V_p K_s\}[P_0]}{K_p(K_s + [S_0])^2 + K_s(K_s + [S_0])[P_0]} \quad (8)$$

and for eqn. (2):

$$\Delta v_0 = \frac{K_s\{V_s(K_{sp} + K_p[S_0])[S_0] + V_p K_{sp}(K_s + [S_0])\}[P_0]}{K_p K_{sp}(K_s + [S_0])^2 + K_s(K_s + [S_0])(K_{sp} + K_p[S_0])[P_0]} \quad (9)$$

A plot of $1/\Delta v_0$ against $1/[P_0]$ at a fixed $[S_0]$ is linear for both Mechanisms I and II.

Mechanisms I and II can, however, be distinguished by analysis of plots of $1/\Delta v_0$ against $1/[P_0]$ at various $[S_0]$ values. Variation in $[S_0]$ may affect the slope of the line and its intercepts on the $1/[P_0]$ axis and on the $1/\Delta v_0$ axis. The simplest parameter to analyse is the intercept on the $1/[P_0]$ axis, I_p. From eqn. (8) I_p is:

$$I_p = -\frac{K_s}{K_p(K_s + [S_0])} \quad (10)$$

and from eqn. (9) it is:

$$I_p = -\frac{K_s(K_{sp} + K_p[S_0])}{K_p K_{sp}(K_s + [S_0])} \quad (11)$$

For Mechanism I [eqn. (10)] one can see that a plot of $-1/I_p$ against $[S_0]$ is linear:

$$-\frac{1}{I_p} = \frac{K_p}{K_s}\cdot[S_0] + K_p \quad (12)$$

whereas for Mechanism II [equation (11)] such a plot is non-linear:

$$-\frac{1}{I_p} = \frac{K_p K_{sp}(K_s + [S_0])}{K_s(K_{sp} + K_p[S_0])} \quad (13)$$

Thus we can conclude that:

(i) if $(-1/I_p)$ varies linearly with $[S_0]$, then the results are consistent with Mechanism I;

(ii) if a plot of $(-1/I_p)$ against $[S_0]$ is non-linear, then the results are consistent with Mechanism II.

Estimation of the Parameter K_{sp}

Taraszka & Alberty (1964) estimated K_{sp} for the reaction of fumarase (L-malate hydro-lyase, EC 4.2.1.2) from a rate equation similar to eqn. (2). Their equation, which took into account the observed activation of the reaction at high $[S_0]$ and $[P_0]$ values, was:

$$v = \frac{\left(\frac{V_s}{K_s}\cdot[S] - \frac{V_p}{K_p}\cdot[P]\right)(1 + \theta_s[S] + \theta_p[P])}{1 + \frac{[S]}{K_s} + \frac{[P]}{K_p} + \frac{[SP]}{K_{sp}} + \frac{[S]^2}{K_{ss}} + \frac{[P]^2}{K_{pp}}} \quad (14)$$

where θ_s, θ_p, K_{ss} and K_{pp} are further kinetic parameters. To estimate K_{sp} they:

(i) obtained estimates of V_s, K_s and θ_s from measurements of $(v_0)_{[P]=0}$ and estimates of V_p, K_p and θ_p from measurements of $(v_0)_{[S]=0}$; they then inserted these values as constants in eqn. (14);

(ii) treated K_{ss} and K_{pp} as infinitely large so that the $[S_0]^2/K_{ss}$ and the $[P_0]^2/K_{pp}$ terms became negligible;

(iii) measured the initial steady-state velocity in the presence of different initial substrate and product concentrations; each set of values for v_0, $[S_0]$ and $[P_0]$ was substituted into eqn. (14) and the corresponding value of K_{sp} was calculated.

This procedure is cumbersome and any errors incurred in estimating the parameters V_s, V_p, K_s, K_p, θ_s and θ_p lead to larger errors in the estimate of K_{sp} (Taraszka & Alberty, 1964).

By using an extension of the graphical procedure described in the previous section, it is possible to estimate K_{sp}. This method is simpler and more accurate than that of Taraszka & Alberty (1964).

A lower or an upper bound can be placed on the magnitude of K_{sp} by observing whether I_p increases or decreases with increasing $[S_0]$. The derivative of I_p with respect to $[S_0]$:

$$\frac{dI_p}{d[S_0]} = \frac{K_s(K_{sp} - K_s K_p)}{K_p K_{sp}(K_s + [S_0])^2} \quad (15)$$

indicates that for all values of $[S_0]$, the slope of a plot of I_p against $[S_0]$ is:

(i) positive, if $K_{sp} > K_s K_p$,
(ii) negative, if $K_{sp} < K_s K_p$,
(iii) zero, if $K_{sp} = K_s K_p$.

A more exact estimate of K_{sp} can be obtained by converting eqn. (15) into a more convenient form by introducing the term ΔI_p, which is defined by:

$$\Delta I_p = (I_p)_{[S_0]=0} - I_p \quad (16)$$

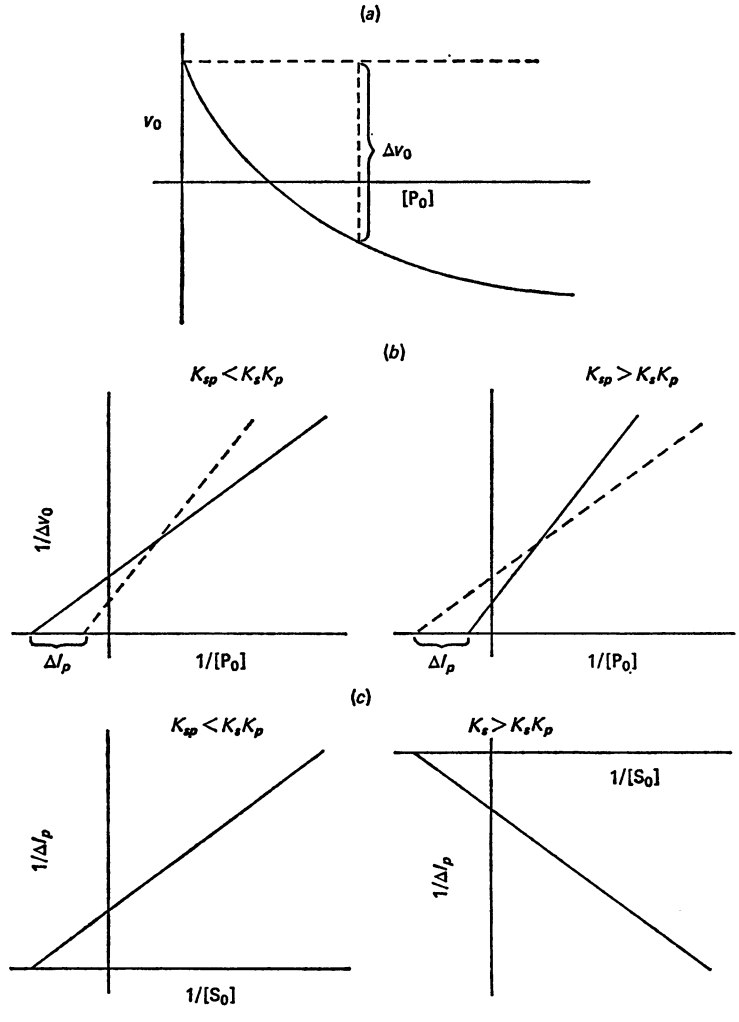

Fig. 1. *Procedure for estimating* K_{sp}

(*a*) A plot of v_0 against $[P_0]$ at a fixed $[S_0]$. This allows values of Δv_0 to be computed for plotting Fig. 1(*b*). (*b*) Plots of $1/\Delta v_0$ against $1/[P_0]$ at a fixed $[S_0] \neq 0$ (——) and when $[S_0] = 0$ (– – – –). This allows values of ΔI_p to be computed for plotting Fig. 1(*c*). (*c*) Plots of $1/\Delta I_p$ against $1/[S_0]$. K_{sp} is obtained by substitution into eqn. (21) of:

(1) G', i.e. the slope of the line in Fig. 1(*c*);
(2) I'_p, i.e. the intercept made by the line on the $1/\Delta I_p$ axis in Fig. 1(*c*);
(3) $(I_p)_{[S_0]=0}$, i.e. the intercept made by the broken line on the $1/[P_0]$ axis in Fig. 1(*b*).

When substrate is absent at the beginning of the reaction, from eqn. (11):

$$(I_p)_{[S_0]=0} = -\frac{1}{K_p} \tag{17}$$

Hence:

$$\Delta I_p = \frac{(K_s K_p - K_{sp})[S_0]}{K_p K_{sp}(K_s + [S_0])} \tag{18}$$

A plot of $1/\Delta I_p$ against $1/[S_0]$ is thus linear. The slope of the line (G') and the intercept on the $1/\Delta I_p$ axis (I'_p) are:

$$G' = \frac{K_s K_p K_{sp}}{K_s K_p - K_{sp}} \tag{19}$$

and

$$I'_p = \frac{K_p K_{sp}}{K_s K_p - K_{sp}} \tag{20}$$

By using eqns. (17), (19) and (20), one can readily calculate K_{sp} from eqn. (21):

$$K_{sp} = \frac{G'}{1 - I'_p (I_p)_{[S_0]=0}} \qquad (21)$$

The procedure for estimating K_{sp} is illustrated in Fig. 1.

Concluding Comment

This graphical procedure is much simpler than the treatment described by Taraszka & Alberty (1964). Unlike the method of Cennamo (1969), it should be useful even when it is not possible to obtain sufficiently high initial substrate concentrations.

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